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14. ABSTRACT
In this study, we generated the replicating Ad5-based vector encoding the Ad41 short fiber devoid of CAR and HSG binding while containing C-terminal His tag for targeting purposes. We produced bispecific 3D5-C6.5 scFv with affinities for both His tag and c-*erb*B-2 oncoprotein. The utility of the 3D5-C6.5 adapter for Ad5F41s6H vector targeting was assessed in c-*erb*B-2-positive cells. Ad5F41s6HsFv and Ad5F41sFv vectors that express the secretory 3D5-C6.5 adapter upon cell infection have been rescued and efficiently propagated while several attempts to rescue their derivatives lacking integrin-dependent cell binding were unsuccessful. The improved capacity of Ad5F41s6HsFv vector producing 3D5-C6.5 to achieve infection of c-*erb*B-2-positive cells was demonstrated by the analysis of Ad replication *in vitro*. However, Ad5F41s6HsFv vector has not demonstrated appreciable changes in the *in vivo* distribution profile compared to Ad5FY477A-6H and Ad5 vectors indicating that additional ablation of integrin-binding is required to retarget Ad vector from normal tissue to the tumor site upon systemic administration.

15. SUBJECT TERMS
Cancer therapy, adenovirus, replication-competent, targeting, bispecific scFv

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INTRODUCTION

Cancer gene therapy has been evaluated as a candidate therapy for a variety of carcinomas to eradicate loco-regional and disseminated disease, which is not adequately addressed by conventional treatments. Viral agents could represent a powerful anticancer treatment platform if they can be designed to infect tumor cells with a requisite level of efficiency and specificity. In this regard, replication-competent adenovirus (Ad) vectors have been of high interest, owing to their ability to propagate in epithelial cells, the origin of most human cancers. However, realization of the full potential of Ad vectors for targeted cancer treatment is currently limited by broad viral tropism which results in widespread tissue distribution of systemically administered Ad with preferential accumulation in the liver (*1*). The ability to control Ad tropism *in vivo* to restrict infection to cancer cells will certainly increase both the safety and efficacy of virus-mediated treatment while expanding the use of Ad vectors beyond loco-regional administration in humans. The achievement of such targeted infection requires both ablation of broad viral tropism and engineering of a novel mechanism of selective cancer cell recognition. In this respect, tropism of widely used Ad of serotype 5 (Ad5) is mediated by interaction of capsid fiber protein with coxsackie-adenovirus receptor (CAR) and heparan sulfate glycosaminoglycans (HSG) as well as by binding of the Arg-Gly-Asp (RGD) motif in penton to α_v-integrins on the cell surface(*2*). Therefore, elimination of virus binding to CAR, HSG, and integrins coupled with the recognition for a tumor-specific antigen would represent a highly promising approach to restrict ectopic infection and to retarget Ad vector to tumor sites. We hypothesize that the replacement of the fiber protein in Ad5 capsid with the short Ad41 fiber, which lacks both CAR and HSG binding (*3*), along with the RGD motif deletion in penton will result in complete elimination of endogenous Ad tropism both *in vitro* and *in vivo*. A new tropism restricted to cancer cells will be conferred by self-association of virus and a paracrine targeting adaptor expressed by this Ad vector, thereby providing binding of the modified viral capsid to a tumor-specific receptor. To this end, we propose to design a bispecific single-chain antibody (scFv) with affinities for the c-*erb*B-2 oncoprotein (*4*), a molecule of established therapeutic relevance in breast cancer, and a six histidine tag (6His) (*5*) genetically incorporated into the carboxy-terminus of short Ad41 fiber protein. Importantly, productive infection of such native tropism-ablated Ad vector should occur solely via association between the c-*erb*B-2-targeting adaptor secreted from infected cells and the progeny virions released upon cell lysis. Thus, the purpose of this proposal is to develop an Ad vector system allowing selective killing of c-*erb*B-2-positive malignant cells and efficient tumor oncolysis while sparing normal tissues. Ultimately, our strategy is expected to result in innovative treatment approach for carcinoma of the breast based on an oncotropic Ad system suited to the mandates of systemic administration.

BODY

Task 1. To engineer a native tropism-ablated Ad vector, incorporating the short Ad41 fiber with 6His tag, which expresses a secretory targeting adapter with affinities for both 6His and c-*erb*B-2 oncoprotein.

a) Engineer the gene coding for the short Ad41 fiber protein containing carboxy-terminal 6His tag sequence.

We chose to engineer the Ad41 short fiber protein to incorporate C-terminal protein purificaion tag consisting of six histidine (His) residues, which has previously been shown to provide specificity for artificial cellular receptor (*6*). To this end the C-terminal of the short fiber protein of an Ad41 was genetically modified by the addition of a short peptide linker followed by six His residues. The DNA sequence encoding the Ad41 short fiber shaft and knob regions was PCR amplified from the Ad41 genome using forward primer 5'-GGA AGA ACC ACC TGG TGT TTT AGC AC-3' containing *Dra*III recognition site and reverse primer 5'-CCA TCG ATT GTT CAG TTA TGT AGC AAA-3' containing *Cla*I site. The PCR product was digested with *Cla*I and *Dra*III and clone into the previously described pNEB.PK3.6 (*7*) digested with *Cla*I and *Dra*III site, which was introduced by PCR into the tail-shaft juncture of the Ad5 fiber sequence. The resultant plasmid plasmid containing the gene for the Ad5 fiber tail fused in frame with the Ad41 short fiber shaft and knob was designated as pNF5t41s and then was used to introduce the C-terminal six-His tag sequence. To construct the gene encoding the Ad41 short fiber protein with a C-terminal six-His tag a duplex made of two oligonucleotides (5′-CGA TAA TAA AGA ATC GTT TGT GTT ATG TTT CAA CGT GTT TAT TTT TC-3′ and 5′-AAT TGA AAA ATA AAC ACG TTG AAA CAT AAC ACA AAC GAT TCT TTA TTA T-3′) was cloned into *Cla*I- and *Mfe*I-cleaved pNF5t41s. This resulted in the addition of a short peptide linker and a six-His containing peptide, Arg-Gly-Ser-His6, to the C-terminal of the short 41 fiber protein. The resultant plasmid pNF5t41s6H, which contained the gene for the Ad5 fiber tail fused in frame with the Ad41 short fiber shaft and knob followed by the C-terminal six-His tag, and previously generated pNF5t41s were used for homologous DNA recombination in *E. coli* with the previously described pVK50 (*8*) in order to generate plasmids carrying the recombinant Ad5 genome containing the modified Ad41s fiber genes.

b) Design the gene encoding a secretory form of bispecific scFv with affinities for both 6His tag and c-*erb*B-2 oncoprotein under the control of cytomegalovirus (CMV) immediate early promoter.

To generate a secretory adapter protein targeting Ad vector to the cancer cells expressing HER-2/*new* oncogene we proposed to construct a bispecific single-chain antibody (BscFv) possessing affinities for both the c-*erb*B-2 oncoprotein and the His tag genetically incorporated into the short Ad41 fiber protein. To construct such bispecific adapter we choose to employ 3D5 scFv, a generous gift from Andreas Plückthun (University of Zurich, Zurich, Switzerland), that recognizes the carboxy-terminal His tag (*5*) and C6.5 scFv specific to c-*erb*B-2 oncoprotein (*4*) kindly provided by by James D. Marks (Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA). To this end we used a mammalian expression vector (pDisplay; Invitrogen) containing the DNA of 3D5 scFv ligated in frame downstream of the murine Ig kappa-chain leader sequence while followed by HA epitope and the transmembrane domain of platelet-derived growth factor receptor (PDGFR) under the transcriptional control of CMV promoter as described previously (*6*). First, this plasmid was modified by deleting PDGFR domain to avoid anchoring the protein to the plasma membrane and inserting instead the oligonucleotide duplex encoding Strep-tag II peptide (WSHPQFEK) (*9*) convenient for protein purification purposes. The DNA sequence encoding C6.5 scFv was PCR amplified from the previously described pFBsCARfC6.5 plasmid (*10*) using forward primer (5'-TCA ATC CGG ACA GGT TCA GCT GGT GCA GTC-3') and reverse primer (5'-GCG GTC CGG ATA GTA CAG TCA GCT TGG TC-3') designed to include *Bsp*EI recognition site. We choose to assemble the gene encoding

3D5-C6.5 BscFv in the following orientation: $3D5V_L$-$C6.5V_H$-V_L-$3D5V_H$. The V_L domain of 3D5 scFv carries a 9-amino acid N-terminal extension (YPYDVPDYA) that is recognized by the anti-HA peptide mAb. The V_H domain of 3D5 scFv carries a 8-amino acid (DYKDDDDK), which is recognized by the anti-Flag mAb, followed by Strep-tag II N-terminal extension. The resultant plasmid was designated as pD3D5-C6.5fs and used further to generate the shuttle plasmid for incorporation of 3D5-C6.5 BscFv into the viral genome.

c) Construct the plasmid shuttle vector for incorporation of the CMV-driven bispecific scFv in place of partially deleted E4 region and the gene for the short Ad41 fiber with the carboxy-terminal 6His tag into the Ad5 genome devoid of fiber and RGD coding sequences.

To construct the shuttle vector for incorporation of 3D5-C6.5 BscFv into the viral genome we used pR-Ad6m plasmid (*11*), which encodes right part of the Ad5 genome including inverted terminal repeats and deleted E4 region except ORF6. The DNA fragment containing the 3D5-C6.5 BscFv gene under the transcriptional control of the CMV promoter and followed by polyA signal was isolated from constructed pD3D5-C6.5fs vector by digestion with *Hind*III and *Xho*I. The isolated DNA fragment containing CMV-3D5-C6.5-pA sequence was cloned in place of deleted E4 region in the pR-Ad6m plasmid digested with *Sal*I and *Hind*III resulting in the shuttle vector designated as pKE4 3D5-C6.5fs.

d) Use constructed shuttle plasmid to generate recombinant viral genome by homologous recombination in E.coli and then rescue native tropism-ablated Ad5dRGDs41F6his vector using U118AR cells displaying an artificial receptor containing an anti-6His scFv.

We choose first to generate Ad5 vector containing the Ad41 short fiber with carboxy-terminal six-His tag, Ad5F41s6H, and control vector Ad5F41s and study their infection properties. To this end, pAd5F41s6H and pAd5F41s plasmids carrying recombinant viral genomes were constructed by homologous DNA recombination in *E. coli* between the newly designed shuttle plasmids and the previously described pVK50 (*8*). pAd5F41s6H and pAd5F41s plasmids were digested with *Pac*I to release viral genomes and were transfected to 211B cells (*12*), which express the Ad5 fiber protein and could complement for the fiber functions. Transfections of 211B cells with recombinant viral genomes have resulted in the rescue of Ad5F41s and Ad5F41s6H vectors. Both Ad vectors were propagated on 293 cells and purified by centrifugation in CsCI gradients by a standard protocol. Viral particle titers were determined spectrophotometrically by the method of Maizel et al (*13*). The identity of the viruses was confirmed by partial sequencing of DNA isolated from purified virions as well as by western blot analysis of viral proteins performed with 4D2 mAb specific to the Ad fiber protein tail portion (*14*) and the anti-His mAb, Penta-His (Qiagen, Valencia, CA).

Western blot analysis of generated Ad vectors using 4D2 mAb has demonstrated that the chimeric Ad41 short fibers of the predicted size are incorporated into their virions (Fig. 1A). The presence of His tag on the fibers of Ad5F41s6H was confirmed by their specific interaction with Penta-His mAb as compared to the fibers of Ad5F41s and Ad5wtY477A-6H control viruses (Fig. 1B).

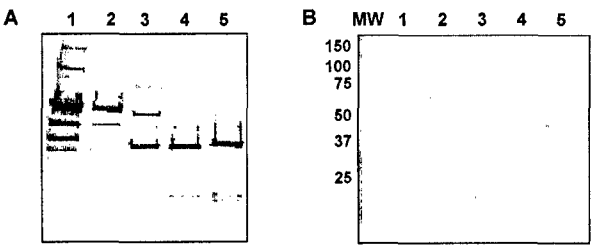

Fig. 1. Western blot analysis of Ad vectors containing the Ad41 short fiber. The Ad5 fiber containing the N-terminal six-His tag produced using baculovirus expression system (lane 1) and samples of CsCl-purified AdwtY477A-6His (lane 2), Ad41 (lane 3), Ad5F41s (lane 4), and Ad5F41s6H (lane 5) containing $1x10^{10}$ vp/lane

were boiled in Laemmli loading buffer and separated on 4-15% gradient SDS gel. Electrophoretically resolved proteins were transferred to PVDF membrane and were probed with 4D2 mAb (A) or Penta-His mAb (B). Bound mAb were then detected with goat anti-mouse antibody-Alkaline phosphatase conjugate (Sigma). The numbers on the left (B) indicate molecular masses of marker proteins (lane MW) in kilodaltons (kDa).

If the His tag incorporated at the C-terminus of the Ad41s fiber is employed for Ad retargeting strategies, then it is of necessity that it is accessible for binding in the context of the intact virion. The accessibility of the His tag in the intact viral particle was verified by the ability of Ad5F41s6H to bind specifically to nickel-nitrilotriacetic acid (Ni-NTA) Agarose (Qiagen, Valencia, CA) (data not shown). To test the ability of the Ad41s fiber-incorporated His tag to mediate Ad infection we used the previously described U118MG-HissFv.rec cells (*6*) that express the cognate artificial receptor, an anti-His tag sFv. Monolayers of the parental U118MG and U118MG-HissFv.rec cells were infected with Ad5F41s6H, Ad5F41s, or previously described AdwtY477A-6His (*15*) at MOIs ranging from 1 to 900 viral particles per cell. AdwtY477A-6His vector has the Ad5 fiber knob domain containing both the mutation (Y477A) ablating binding to CAR and the C-terminal His tag. Eight days after infection, the CPE was monitored by staining the viable cells with crystal violet. As shown in Fig. 2, Ad5F41s6H caused extensive cytopathic effect in the U118MG-HissFv.rec cells at an MOI of 33 vp/cell and almost completely lysed the cells at an MOI of 100 vp/cell. In contrast, more then 100 vp/cell of Ad5F41s6H were required to induce a comparable CPE in U118MG cells while Ad5F41s and AdwtY477A-6His failed to completely kill either cell line even at an MOI of 900. Preincubation of Ad5lucFc6HIS with an anti-His mAb inhibited infection of the U118MG.HissFv.rec cells in a dose-dependent manner (data not shown). This indicates that the engineered Ad5Fs6H virus infected U118MG.HissFv.rec cells by means of a specific interaction between the Ad41s fiber-incorporated His tag and the cognate anti-His sFv artificial receptor. In addition, these data demonstrated the feasibility of employing the anti-His tag sFv as a component of bispicific Ad-targeting adapter protein.

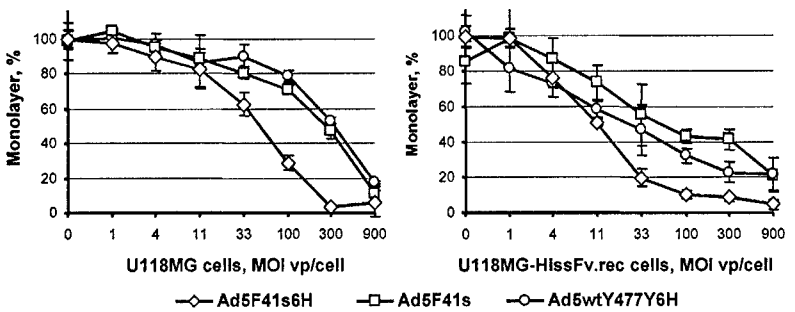

Fig. 2. Propagation of Ad5F41s6H vector in U118MG-HissFv.rec cells. Monolayers of U118MG-HissFv.rec and the parental U118MG cells were infected with Ad5F41s6H, Ad5F41s, or AdwtY477A-6His at the indicated MOI or mock infected. Eight days post-infection, the CPE was monitored by staining the viable cells with crystal violet and the stained monolayers were scanned using multi-functional Synergy HT plate reader (Bio-Tek Instruments, Inc.) set at 565 nm. The data point is presented as the percentage of infected monolayer integrity with respect to the uninfected control. Each point represents the cumulative mean ±SD of triplicate determinations.

Task 2. To assess oncolytic effects of this c-*erb*B-2-targeted Ad vector system in breast cancer cells *in vitro*.

a) Evaluate the ability of Ad5dRGDs41F6his vector to express a secretory bispecific scFv protein upon infection of U118AR cells using Western blot analysis.

To validate a secretory bispecific scFv protein production in a mammalian system HEK293 cells were transfected with constructed pD3D5-C6.5fs expression vector using Superfect (Qiagen) according to the manufacturer's protocol. The stably transfected cells stably expressing the 3D5-C6.5 were

selected in the presence of G418 at a concentration of 1 mg/ml. The 3D5-C6.5 protein secreted from trasfected cells was detected in culture media by binding to the ErbB2/Fc chimera (R&D Systems, Inc.) in ELISA as described below in (b). The secretory 3D5-C6.5 scFv was purified from culture media by affinity chromatography on Strep-Tactin resin (Sigma-Genosys; St Louis, MO), which binds Strep-tag II-containing proteins. To characterize the polypeptide composition of soluble 3D5-C6.5 fusion protein western blot analysis was performed with anti-Flag M2 mAb, ani-HA epitope mAb (Sigma; St Louis, MO), and alkaline phosphatase-conjugated Strep-Tactin (Bio-Rad; Hercules, CA) specific to Strep-tag II. Detection of denatured 3D5-C6.5 scFv resolved by SDS-PAGE using specific antibodies revealed the presence of both the N-terminal FLAG and the C-terminal HA epitope in the context of major protein band with molecular mass of 57 kDa, which was expected for monomeric form of 3D5-C6.5 scFv molecule (Fig. 3). Thus, the analysis of purified 3D5-C6.5 protein indicates that generated bispecific scFv maintained the designed composition and relatively stable protein structure.

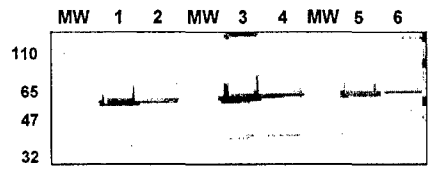

Fig. 3. Western blot analysis of 3D5-C6.5 fusion protein. Samples of purified 3D5-C6.5 protein were loaded onto 4-15% gradient SDS gel in Laemmli buffer. Samples in lanes 2, 4, and 6 were boiled prior to loading to denature the protein complexes while samples in lanes 1, 3, and 5 were not boiled. Electrophoretically resolved proteins were transferred to PVDF membrane and were probed with Alkaline phosphatase-conjugated anti-Flag M2 mAb (lanes 1, 2), ani-HA epitope mAb (Lanes 3, 4) (Sigma), and Alkaline phosphatase-conjugated Streptactin (lanes 5, 6) (Bio-Rad). The numbers on the left indicate molecular masses of marker proteins (MW) in kilodaltons (kDa).

b) Purify an scFv adapter protein secreted from U118AR cells infected with Ad5dRGDs41F6his vector by affinity chromatography and confirm its binding specificity for both the cellular c-*erb*B-2 oncoprotein and the 6His tag displayed on Ad41 fiber.

The secretory 3D5-C6.5 fusion protein was purified from culture media as described above and its binding specificity to c-*erb*B-2 oncoprotein and C-terminal His tag was validated in several assays. First, to test the 3D5-C6.5 binding specificity to the Ad41s fiber C-terminal His tag we used western blot analysis of Ad5F41s6H proteins probed with 3D5-C6.5. It was shown that 3D5-C6.5 scFv fusion specifically detected the C-terminal His tag in fiber proteins of Ad5F41s6H and Ad5wtY477A-6H viruses but not in the control Ad41 and Ad5F41s viruses (Fig. 4).

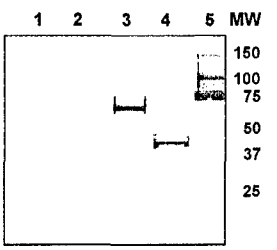

Fig. 4. Analysis of 3D5-C6.5 recognition of fiber-incorporated His tag. Samples of CsCl-purified Ad41 (lane 1), Ad5F41s (lane 2), AdwtY477A-6His (lane 3) and Ad5F41s6H (lane 4) containing $1x10^{10}$ vp/lane were boiled in Laemmli loading buffer and separated on 4-15% gradient SDS gel. Electrophoretically resolved proteins were transferred to PVDF membrane and were probed with 3D5-C6.5 BscFv. Bound BscFv were then detected with

anti-Flag M2 mAb-Alkaline phosphatase conjugate (Sigma). The numbers on the right indicate molecular masses of marker proteins (lane 5) in kilodaltons (kDa).

To validate Ad41s fiber-incorporated His tag accessibility in the context of the intact virion we analyzed binding of 3D5-C6.5 adapter to the absorbed Ad5F41s6H virions in ELISA. As shown in Fig. 5, Ad5F41s6H virions demonstrated a dose-dependent binding to the 3D5-C6.5 fusion protein while there was no binding detected between 3D5-C6.5 and the control Ad5F41s virions. These data clearly demonstrated the specific recognition of fiber-incorporated His tag in the context of intact Ad5F41s6H virions by generated 3D5-C6.5 adapter protein.

The specificity of 3D5-C6.5 adapter interaction with c-*erb*B-2 oncoprotein was evaluated in ELISA using the ErbB2/Fc chimeric protein (R&D Systems Inc.) as an antigen. Purified 3D5-C6.5 showed a dose-dependent binding to the erbB2/Fc chimera demonstrating that C6.5 scFv incorporated in the context of fusion protein retained its specificity for c-*er*bB-2 oncoprotein (Fig. 6). Thus, we confirmed the ability of constructed 3D5-C6.5 adapter protein to maintain distinct binding specificities towards both the C-terminal His tag and the c-*erb*B-2 oncoprotein in a single molecule design.

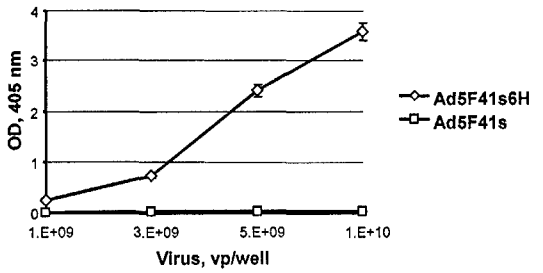

Fig. 5. Analysis of 3D5-C6.5 protein binding to Ad5F41s6H virions. Ad5F41s6H and Ad5F41 virions were adsorbed on Nunc-Maxisorp ELISA plate at the indicated concentrations and probed with purified 3D5-C6.5 protein at the concentration of 50 ng/ml. Bound 3D5-C6.5 protein was detected using anti-Flag M2 mAb-Alkaline phosphatase conjugate (Sigma). The plate was developed with p-nitrophenyl phosphate and read in Synergy HT plate reader (Bio-Tek Instruments, Inc.) set at 405 nm. Each point represents the cumulative mean ±SD of triplicate determinations.

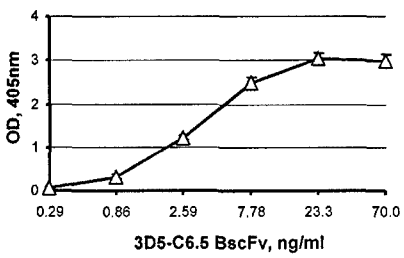

Fig. 6. Analysis of 3D5-C6.5 BscFv binding to erbB-2. The erbB2/Fc chimeric protein (R&D systems) expressed in mammalian system was adsorbed on Nunc-Maxisorp ELISA plate at the concentration of 50 ng/well and probed with serial dilutions of purified 3D5-C6.5 scFv. Binding was detected using anti-HA epitope mAb conjugated with alkaline phosphatase (Sigma). The plate was developed with p-nitrophenyl phosphate and read in a microtiter plate reader set at 405 nm. Each point represents the cumulative mean ±SD of triplicate determinations.

Having shown bispecific binding properties of our 3D5-C6.5 scFv-derived adapter protein, we evaluated its ability to target infection of Ad vector containing His-tagged fiber to the c-*erb*B-2-positive breast cancer cells. This assay was carried out using 435.eB1 breast cancer cells stably transformed to express c-*erb*B-2 (*16*) and the parental MDA-MB-435 breast adenocarcinoma cells (*17*), which were kindly provided by Dr. Dihua Yu (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Since our replication-competent Ad5F41s6H vector was not designed to contain a marker gene, for the proof of principle we employed the previously described AdGFPLucY477A-6His (*15*) to

characterize the c-*erb*B-2-targeting properties of 3D5-C6.5 adapter. AdGFPLucY477A-6His vector has the Ad5 fiber containing both the mutation (Y477A) ablating CAR binding and the C-terminal His tag while encoding firefly luciferase and GFP marker genes in deleted E1A region. To determine the improvements of AdGFPLucY477A-6His infection efficiency the 3D5-C6.5 protein was tittered against a constant dose of AdGFPLucY477A-6His (100 vp/cell) and relative luciferase activity was measured in infected cells. Hence, we compared the magnitude of gene transfer augmentation provided by the 3D5-C6.5 targeting adapter complexed with either AdGFPLucY477A-6His or AdGFPLuc virus lacking fiber-incorporated His tag. Fig. 7 illustrates the 3D5-C6.5 dose-dependent enhancement of Ad-mediated gene transfer to 435.eB1 and MB435 cells that was achieved by targeted AdGFPLucY477A-6His compared to the control AdGFPLuc vector. As shown in Fig. 7, the 3D4-C6.5-targeting protein provided a 4.3-fold increase of AdGFPLucY477A-6His infection efficiency in 435.eB1 cells compared to MB435 cells. Importantly, the use of the 3D4-C6.5 adapter complexed with control AdGFPLuc vector did not result in any infection augmentation in both 435.eB1 and MB435 cell cultures. These data strongly indicate that the bispecific 3D4-C6.5-targeting adapter promoted the AdGFPLucY477A-6His infection of CAR-deficient cells specifically via a c-*er*bB-2-dependent pathway.

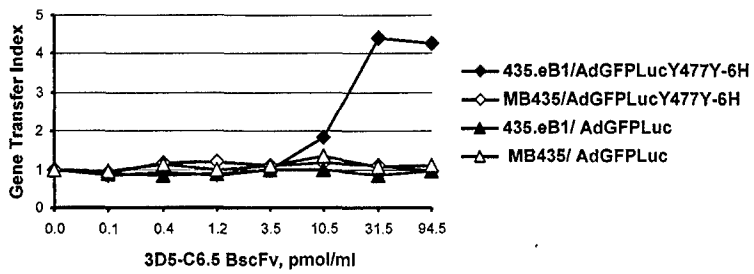

Fig. 7. 3D5-C6.5 improves AdGFPLucY477A-6His infection of c-*erb*B-2-positive cells. Purified 3D5-C6.5 BscFv was preincubated at varying concentrations with either AdGFPLuc or AdGFPLucY477A-6His vector expressing luciferase reporter gene. Monolayers of MB435 or 435.eB1 cells were exposed to the virus-BscFv mixtures or virus alone at an MOI of 100 vp/cell. Cells were incubated for 48 h to allow expression of reporter gene, lysed, and the luciferase activity was measured. Results are presented as gene transfer indexes that were calculated as the ratio of luciferase activities detected in the cells infected with virus-BscFv complex to luciferase activities in the cells infected with virus alone. Each point represents the cumulative mean ±SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

c) Demonstrate the capacity of Ad5dRGDs41F6his vector to achieve infection of c-*erb*B-2-positive breast cancer cells mediated by a secretory targeting scFv adapter *in vitro* by analysis of cytotoxicity and virus yield.

Prior to testing the capacity of Ad5F41s6H to achieve infection of c-*erb*B-2-positive breast cancer cells mediated by the 3D5-C6.5 adapter we choose to characterize the oncolytic potential of this replicating virus in 293AR cells expressing the cognate artificial receptor, an anti-His tag sFv (*6*). In this assay we compared a relative cell-killing ability of our Ad5F41s6H vector with Ad5F41s, CAR tropism-ablated AdwtY477A-6His replication-competent virus, and wild type Ad5. The cytopathic effect induced by virus propagation was monitored by staining the viable cells with crystal violet and the degree of cell monolayer loss was compared between the viruses at the same MOI. We found that while the parental 293 cells were relatively refractory to Ad5F41s6H, this virus was more infectious in 293AR cells as compared to the control Ad5F41s vector (Fig. 8). Both 293 and 293AR cells revealed similar Ad5wt infectivity while only 293AR cells could efficiently maintain propagation of CAR tropism-ablated Ad5F41s6H and AdwtY477A-6His vectors. These results clearly demonstrate that generated Ad5F41s6H indeed lacks CAR-mediated tropism while is able to utilize fiber-incorporated His tag for cell binding.

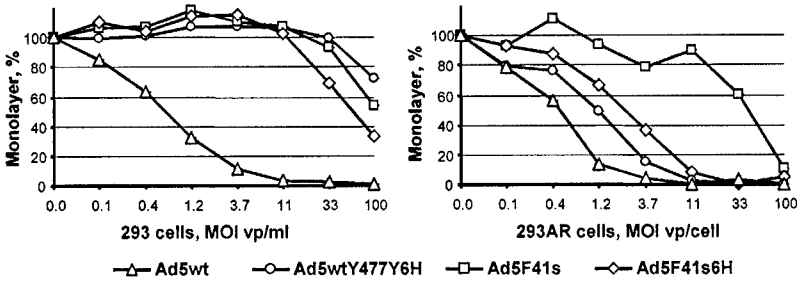

Fig. 8. Analysis of Ad5F41s6H infection efficiency in 293AR cells. Monolayers of 293AR and the parental 293 cells were infected with Ad5 wt, AdwtY477A-6His, Ad5F41s, or Ad5F41s6H at the indicated MOI or mock infected. Four days post-infection, the CPE was monitored by staining the viable cells with crystal violet and the stained monolayers were scanned using multi-functional Synergy HT plate reader (Bio-Tek Instruments, Inc.) set at 565 nm. The data point is presented as the percentage of infected monolayer integrity with respect to the uninfected control. Each point represents the cumulative mean ±SD of triplicate determinations.

We then examined the utility of the 3D5-C6.5 adapter for Ad5F41s6H vector targeting by assessing its ability to improve oncolysis of c-*er*bB-2-positive 435.eB1 breast cancer cells. Monolayers of MB435 and 435.eB1 cells were infected with Ad5F41s6H, Ad5F41s, AdwtY477A-6His, or Ad5wt at the varying MOIs (1 - 900 vp/cell) in the presence of 3D5-C6.5 adapter protein (30 pmol/ml) and the CPE was monitored by cell staining with crystal violet. Four days post-infection, significantly increased cytopathic effect could be observed in 435.eB1 cells infected with Ad5F41sH compared to the control viruses and MB435 control cells (Fig. 9).

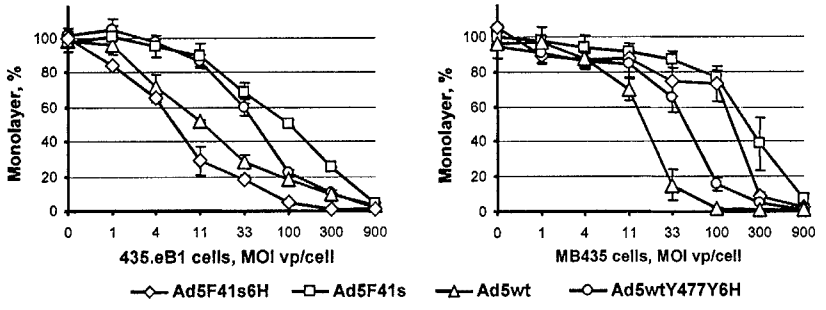

Fig. 9. 3D5-C6.5 improves Ad5F41s6H-mediated oncolysis of c-*erb*B-2-positive cells. Monolayers of 435.eB1 and the parental MB435 cells were infected with Ad5F41s6H, Ad5F41s, Ad5 wt or AdwtY477A-6His in the presence of 3D5-C6.5 BscFv (30 pmol/ml) at the indicated MOI or mock infected. Four days post-infection, the CPE was monitored by staining the viable cells with crystal violet and the stained monolayers were scanned using multi-functional Synergy HT plate reader (Bio-Tek Instruments, Inc.) set at 565 nm. The data point is presented as the percentage of infected monolayer integrity with respect to the uninfected control. Each point represents the cumulative mean ±SD of triplicate determinations.

Hence, the presence of the c-*er*bB-2-targeting 3D5-C6.5 adapter in the infection medium significantly improved the oncolytic potency of the replicating Ad5F41s6H vector *in vitr*o. These results indicated that the 3D5-C6.5 adapter mediated specific interaction between His tag-containing Ad5F41s6H virus and c-*er*bB-2-oncoprotein displayed on 435.eB1 cells allowing more efficient lateral infection and spread the viral progeny throughout cell monolayer.

In order to generate Ad5F41s6H vector expressing 3D5-C6.5 sFv we have used the pR-Ad6m shuttle vector (*11*), kindly provided by Dr. J.Dong (Medical University of South Carolina, Charleston, SC), to incorporate the CMV promoter-driven 3D5-C6.5 gene into the early E4 region which was deleted for all genes except ORF6/7 essential for virus replication. Constructed shuttle vector, pKE4cmv3D5-C6.5, was employed for homologous recombination in E.coli with genomic DNAs of Ad5F41s6H and

Ad5F41s to generate plasmids carrying the Ad5F41s6HsFv and Ad5F41sFv genomes respectively. These plasmids were further employed to introduce the integrin-binding ablating mutation (RGD to RAE) in the penton base of Ad5F41s6HsFv and Ad5F41sFv using shuttle vector designed in our laboratory and kindly provided by Dr. Ramon Alemany (unpublished data). All generated viral genomes were validated for the correct structure of modified regions and inserted sequences and were then transfected to 293AR cells expressing artificial viral receptor facilitating the rescue of Ad vectors lacking native tropism (*6, 18*). Both Ad5F41s6HsFv and Ad5F41sFv have been rescued and efficiently propagated while several attempts to rescue their derivatives lacking integrin-dependent cell binding were unsuccessful. Expression of the secretory 3D5-C6.5 bispecific scFv during propagation of Ad5F41s6HsFv and Ad5F41sFv vectors in cell culture was confirmed as described previously in the Task 2, a) (data not shown).

To evaluate whether virus targeting mediated by the secretory 3D5-C6.5 sFv improves infection of Ad5F41s6HsFv vector with respect to c-*erb*B-2-positive cells we carried out the analysis of Ad replication. To assess the efficiency of viral DNA replication monolayers of MB-435 and 435.eB1 breast cancer cells were infected with Ad5F41s6HsFv, Ad5F41sFv, or wild type Ad5 vector at an MOI of 20 vp/cell and viruses were allowed to propagate for 11 days. Cells were harvested at different time points postinfection and total DNA was purified using QIAamp DNA Blood Kit (QIAGEN, Valencia, CA). The levels of viral DNA amplification were determined by quantitative PCR with primers specific for the E1 genome region using LightCycler System (Roche Diagnostics, Indianapolis, IN). Resultant Ad genomic DNA copy numbers were normalized by amount of DNA in each sample and are presented in Fig. 10. The results of this experiment demonstrated that the efficacy of Ad5F41s6HsFv vector replication was increased 3-fold in c-*erb*B-2-positive 435.eB1 cells compared to MB-435 cells. Replication of wild type Ad5 and Ad5F41sFv control vector was somewhat increased (less than 2-fold) in MB-435 and 435.eB1 cells respectively. These data demonstrated the improved capacity of Ad5F41s6HsFv vector producing secretory targeting adapter, 3D5-C6.5 bispecific scFv, to achieve infection of c-*erb*B-2-positive cells *in vitro* as compared to both control Ad vectors and c-*erb*B-2-deficient cells.

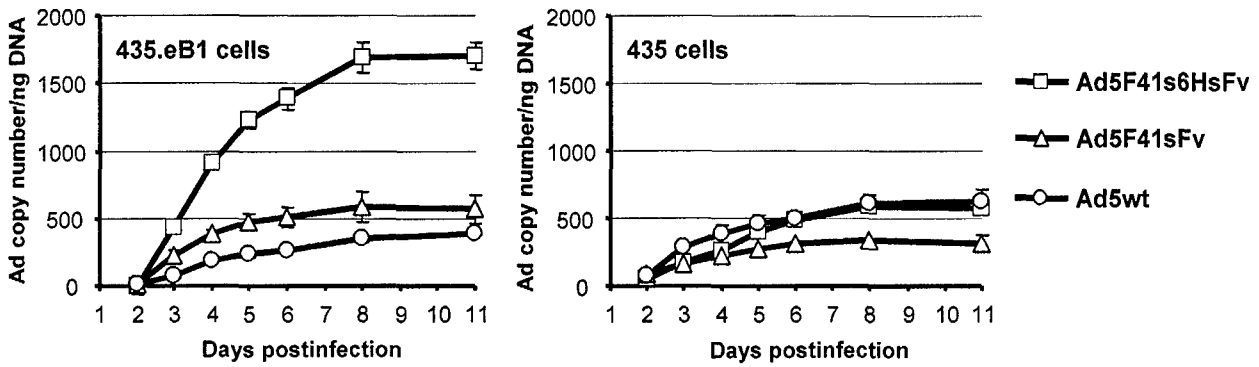

Fig. 10. Analysis of Ad replication efficiency in breast cancer cells. Monolayers of 435.eB1 cells stably expressing c-*erb*B-2 oncoprotein and c-*erb*B-2-deficient MDA-MB-435 cells were infected with Ad5F41s6HsFv, Ad5F41sFv, or wild type Ad5 vector at an MOI of 20 vp/cell. Cells were harvested at the indicated time points postinfection and the levels of viral genome amplification were determined by quantitative PCR. The data are presented as Ad genome copy number normalized by the amount of DNA content in each sample. Each data point represents the cumulative mean $\pm$SD of triplicate determinations.

Task 3. To test therapeutic utility of this c-*erb*B-2-targeted Ad vector system after systemic administration in a murine model of human breast tumor xenografts.

The ultimate preclinical test of an experimental therapeutic is an animal model. To test the therapeutic utility of generated Ad5F41s6HsFv vector for treatment of carcinoma of the breast we studied if our Ad

tropism modification maneuvers resulted in augmented virus ability to infect c-*erb*B-2-positive cells in the stringent setting of a murine model of human tumor xenografts. In order to determine whether optimized Ad tropism alterations were achieved to accomplish targeted infection of cancer cells while sparing normal tissues we analyzed the biodistribution profile of Ad5F41s6HsFv vector after systemic vascular delivery compared to CAR-binding ablated Ad5FY477A-6H, wild type Ad5 and Ad41 controls. All animal methods and procedures were carried out in accordance with Institutional Animal Care and Use Committee guidelines. A murine model of human breast tumor xenografts was created by injecting 10^7 of 435.eB1 cells subcutaneously into each flank of NIH III Nude mouse. Tumors were allowed to grow up to 100 mm^3 and mice were injected into the tail vein with $5x10^{10}$ viral particles of Ad5F41s6HsFv, Ad5FY477A-6H, Ad5, or Ad41 (n = 5 mice/group). Two hours after virus injection mice were sacrificed, tumor nodules along with major organs (liver, lungs, heart, spleen, and kidney) were surgically harvested and mechanically homogenized for purification of total DNA using DNeasy Tissue Kit (QIAGEN, Valencia, CA) and 10 mg of tissue as recommended by the manufacturer. The levels of Ad vector uptake by normal and tumor tissues was determined by quantitative PCR analysis and presented as the percentage of viral genome copies detected at each site calculated with respect to total number of Ad genomic DNA. As can be seen from Ad vector biodistribution data shown in Fig. 11 Ad5F41s6HsFv vector has not demonstrated any appreciable changes in the *in vivo* distribution profile compared to Ad5FY477A-6H (Fig. 11A) and wild type Ad5 vector (Fig. 11B).

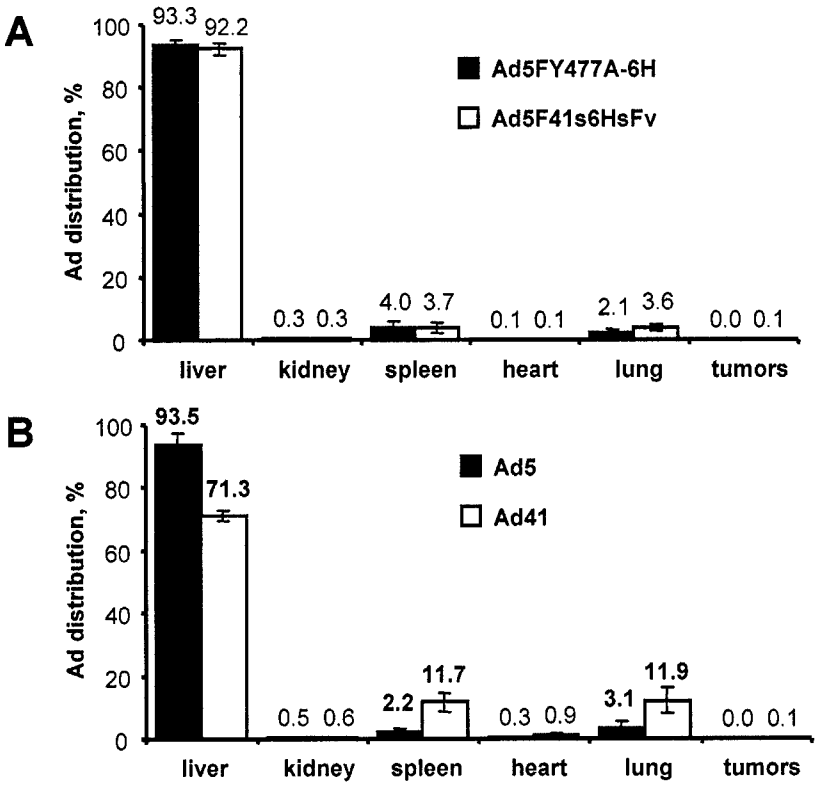

Fig. 11. Ad biodistribution analysis. NIH III nude mice bearing subcutaneous tumor xenografts established using 435.eB1 breast adenocarcinoma cells were injected in to the tail vein with $5x10^{10}$ vp (n = 5 mice/group) of Ad5F41s6HsFv or Ad5FY477A-6H **(A)**, Ad5 or Ad41 **(B)**. Animals were sacrificed two hours postinjection, tumor nodules and major organs were harvested and homogenized for purification of total DNA. The levels of Ad vector uptake by normal and tumor tissues was determined by quantitative PCR using primers specific for the E1 genome region and presented as the percentage of viral genome copies detected at each site calculated with respect to total number of Ad genomic DNA. Each data point represents the cumulative mean $\pm$SD. Statistical significance was tested using two-tailed Students t-test and statistically significant data differences are presented in bold.

Although viral DNA was detected in some tumor xenografts we could not reveal significant differences in the efficiency of virus delivery to the tumor sites between studied Ad vectors. Importantly that Ad41, which is able of CAR binding via long fiber (*19*) but lacks the integrin-binding RGD motif, has demonstrated 20% reduction in virus sequestration by the liver while showing 5- and 4-fold increase of virus uptake in spleen ($p<0.01$) and lung ($p<0.05$) as compared to Ad5 vector respectively (Fig. 11B). These results corroborate the previously published data that CAR-binding ablation alone is not sufficient to change Ad biodistribution *in vivo* (*15*) and additional ablation of integrin binding is required to detarget the virus from normal tissue upon systemic administration in mice and rats (*20, 21*).

KEY RESEARCH ACCOMPLISHMENTS

- The plasmid pD3D5-C6.5fs encoding secretory form of bispecific scFv with affinities for both His tag and c-*erb*B-2 oncoprotein was constructed and HEK293 cell clone stably expressing the gene for 3D5-C6.5 BscFv was selected.
- The secretory 3D5-C6.5 adapter protein was purified and its binding specificity was confirmed for both Ad41s fiber-displayed His tag and c-*erb*B-2 oncoprotein.
- The plasmid pNF5t41s6H containing the gene for the Ad5 fiber tail fused in frame with the Ad41 short fiber shaft and knob followed by the C-terminal six-His tag was constructed. The plasmid pNF5t41s containing the control gene for the Ad5 fiber tail fused in frame with the Ad41 short fiber shaft and knob was constructed.
- The recombinant viral genomes were generated using pNF5t41s6H and pNF5t41s plasmids resulting in rescue of native tropism-ablated Ad5F41s6H and control Ad5F41s vector.
- The oncolytic potential of Ad5F41s6H vector was assessed by analysis of cytotoxicity in U118MG-AR and 293AR cells expressing the cognate artificial receptor.
- The ability of Ad5F41s6H vector to achieve infection of c-*erb*B-2-positive breast cancer cells mediated by the 3D5-C6.5 targeting adaptor was demonstrated *in vitro* by the cytotoxicity assay.
- The improved capacity of Ad5F41s6HsFv vector producing secretory targeting adapter, 3D5-C6.5 bispecific scFv, to achieve infection of c-*erb*B-2-positive cells was demonstrated by the analysis of Ad replication *in vitro*.
- Ad5F41s6HsFv vector has not demonstrated appreciable changes in the *in vivo* distribution profile compared to both Ad5FY477A-6H and wild type Ad5 indicating that CAR-binding ablation alone is not sufficient and additional ablation of integrin binding is required to retarget Ad vector from normal tissue to the tumor site upon systemic administration.

REPORTABLE OUTCOMES

The plasmids that have resulted from this research are the following: pD3D5-C6.5fs, pKE4 3D5-C6.5fs, pKE4cmv3D5-C6.5, pNF5t41s6H, pNF5t41s, pAd5F41s6H, pAd5F41s, pAd5F41s6HsFv and pAd5F41sFv. The recombinant Ad vectors that were generated in this study are Ad5F41s6H, Ad5F41s, Ad5F41s6HsFv, and Ad5F41sFv. HEK293 cell line stably expressing the 3D5-C6.5 BscFv adapter protein was developed in this research.

LIST OF PERSONNEL

1. DMITRIEV, IGOR P., Ph.D. – PI.
2. KASHENTSEVA, ELENA A. – Technical assistant.

CONCLUSIONS

In this study, we have generated the replicating Ad5-based vector encoding the Ad41 short fiber devoid of CAR and HSG binding ability while incorporating the C-terminal His tag for targeting purposes. First, We have compared our Ad5F41s6H vector with control Ad5F41s, CAR tropism-ablated AdwtY477A-6His, and wild type Ad5 for the ability to induce cytopathic effect in CAR-positive 293 cells. We have found that while the parental 293 cells were relatively refractory to Ad5F41s6H, this virus was significantly more infectious in 293AR cells, which express the cognate artificial anti-His tag receptor, as compared to the control Ad5F41s vector. The similar results were obtained using CAR-negative U118MG-AR cells expressing anti-His tag receptor indicating that generated Ad5F41s6H indeed lacks CAR-mediated tropism while is able to utilize fiber-incorporated His tag for cell binding.
In parallel, we designed bispecific 3D5-C6.5 scFv molecule with affinities for both His tag and c-*erb*B-2 oncoprotein. We have confirmed that 3D5-C6.5 BscFv was produced in a secretory form upon expression in mammalian cells and was capable of specific recognition of the Ad41s fiber-incorporated His tag and the extracellular c-*erb*B-2 oncoprotein. We then examined the utility of the 3D5-C6.5 adapter for Ad5F41s6H vector targeting by assessing the adapter's ability to improve oncolysis of c-*er*bB-2-positive cells. The presence of the c-*er*bB-2-targeting 3D5-C6.5 adapter in the infection medium significantly improved the oncolytic potency of the replicating Ad5F41s6H vector in 435.eB1 breast cancer cells compared to c-*er*bB-2-negative MB435 cells. These results indicated that the 3D5-C6.5 adapter mediated specific interaction between Ad5F41s6H virus and the cellular c-*er*bB-2-oncoprotein allowing efficient oncolytic infection and, thus, demonstrated the feasibility of our Ad targeting approach *in vitro*. Ad5F41s6HsFv and Ad5F41sFv vectors that express the secretory 3D5-C6.5 targeting adapter upon cell infection have been rescued and efficiently propagated while several attempts to rescue their derivatives lacking integrin-dependent cell binding were unsuccessful. The improved capacity of Ad5F41s6HsFv vector producing 3D5-C6.5 targeting adapter to achieve infection of c-*erb*B-2-positive cells was demonstrated by the analysis of Ad replication *in vitro*. However, Ad5F41s6HsFv vector has not demonstrated appreciable changes in the *in vivo* distribution profile compared to both Ad5FY477A-6H and wild type Ad5 indicating that CAR-binding ablation alone is not sufficient and additional ablation of integrin binding is required to retarget Ad vector from normal tissue to the tumor site upon systemic administration.

In conclusion, the work presented in this final report completely addressed Task 1, 2, and Task 3 (a) outlined in the approved Statement of Work.

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